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Shugoshin 2 Regulates Localization of the Chromosomal Passenger Proteins in Fission Yeast Mitosis[D]

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Fission yeast has two members of the Shugoshin family, Sgo1 and Sgo2. Although Sgo1 has clearly been established as a protector of centromere cohesion in meiosis I, the roles of Sgo2 remain elusive. Here we show that Sgo2 is required to ensure proper chromosome biorientation upon recovery from a prolonged spindle checkpoint arrest. Consistent with this, Sgo2 is essential for maintaining the Passenger proteins on centromeres upon checkpoint activation. Interestingly, lack of Sgo2 has a more penetrant effect on the localization of Survivin than on the two other Passenger proteins INCENP and Aurora B, and the Survivin-INCENP complex but not the INCENP-Aurora B complex is destabilized in the absence of Sgo2. Finally we show that the conserved C-terminus of Sgo2 is crucial to maintain Sgo2 and Passenger proteins localization on centromeres upon prolonged checkpoint activation. Taken together, our results demonstrate that Sgo2 is important for chromosome biorientation and that it controls docking of the Passenger proteins on chromosomes in early mitotic cells.

INTRODUCTION

To ensure the accuracy of chromosome segregation in mitosis, duplicated sister-chromatids must attach their kinetochores to microtubules emanating from opposite poles, a process referred to as chromosome biorientation. A single mal-orientated chromosome can be recognized by the spindle checkpoint that will block anaphase onset by inhibiting the activity of the anaphase-promoting complex (APC/C). Once all chromosomes are properly biorientated on the metaphase spindle, the spindle checkpoint is silenced and cells proceed through anaphase (for review, see Pinsky and Biggins, 2005).

One of the best characterized roles of the kinase Aurora B is to correct defective kinetochore-microtubule attachment before anaphase onset and therefore ensure proper chromosome biorientation (Tanaka *et al.*, 2002; Ditchfield *et al.*, 2003; Hauf *et al.*, 2003; Tanaka, 2005; Pinsky *et al.*, 2006). Aurora B is also crucial for the recruitment of some spindle checkpoint components to kinetochores (Ditchfield *et al.*, 2003; Vigneron *et al.*, 2004). Thus Aurora B regulates both the physical connections of chromosomes onto the spindle and mitotic progression. When Aurora B activity is compromised, chromosomes mis-segregate massively, leading to aneuploidy (reviewed in Giet *et al.*, 2005). Defective kinetochore-microtubules attachments accumulate in these cells and chromosomes never reach a proper metaphase plate (Hauf *et al.*, 2003).

Aurora B is one of the Chromosomal Passenger proteins, first identified in vertebrates as proteins sharing a complex and highly regulated localization pattern in mitosis (Earnshaw and Bernat, 1991). In particular they transfer abruptly from the inner-centromeres to the spindle midzone at the metaphase to anaphase transition. Each Chromosomal Passenger protein, Survivin, Borealin, TD60, INCENP, and Aurora B has long been recognized as major regulators of mitosis (see Vagnarelli and Earnshaw, 2004 for review). In fission yeast, homologues of only three of the Chromosomal Passenger proteins have been identified, Bir1/Survivin, Pic1/INCENP, and Ark1/Aurora B (Morishita *et al.*, 2001; Petersen *et al.*, 2001; Leverson *et al.*, 2002; Huang *et al.*, 2005). Their localization pattern is also carefully regulated and closely resembles their vertebrate counterparts (see below) and they are also considered as crucial players of mitosis in fission yeast. The mechanisms ensuring the localization and the transfer of the Passenger proteins from one location to another are still poorly understood, as are their exact roles at each location they visit in the cell.

The Shugoshin proteins were first identified as regulators of chromosome segregation in *Drosophila* meiosis (MEI-S332) and have since fuelled a great deal of interest (reviewed in Watanabe and Kitajima, 2005). Shugoshin 1 (Sgo1) protects cohesion at centromeres in the pre-anaphase stages of meiosis I, partly by regulating cohesin phosphorylation status through the recruitment of a specific phosphatase to centromeres (Kitajima *et al.*, 2006; Riedel *et al.*, 2006; Tang *et al.*, 2006). Similarly, homologues of Sgo1 help protect centromeric cohesion during the pre-anaphase stages of vertebrate mitosis (reviewed in Watanabe, 2005). However, the sole budding yeast Shugoshin (ScSgo1) is required to maintain sister chromatid cohesion in meiosis I but not in mitosis (Katis *et al.*, 2004; Marston *et al.*, 2004; Indjeian *et al.*, 2005), demonstrating that Shugoshin function in centromere cohesion is not universal in mitosis. Other roles have been ascribed to the Shugoshin proteins, such as monitoring tension between sister-chromatids (Indjeian *et al.*, 2005) and regulating microtubules dynamics (Salic *et al.*, 2004; Suzuki *et al.*, 2006).

Fission yeast has two members of the Shugoshin family, Sgo1 and Sgo2. Sgo1 appears to have only meiotic functions, whereas lack of Sgo2 triggers both meiotic and mitotic de-

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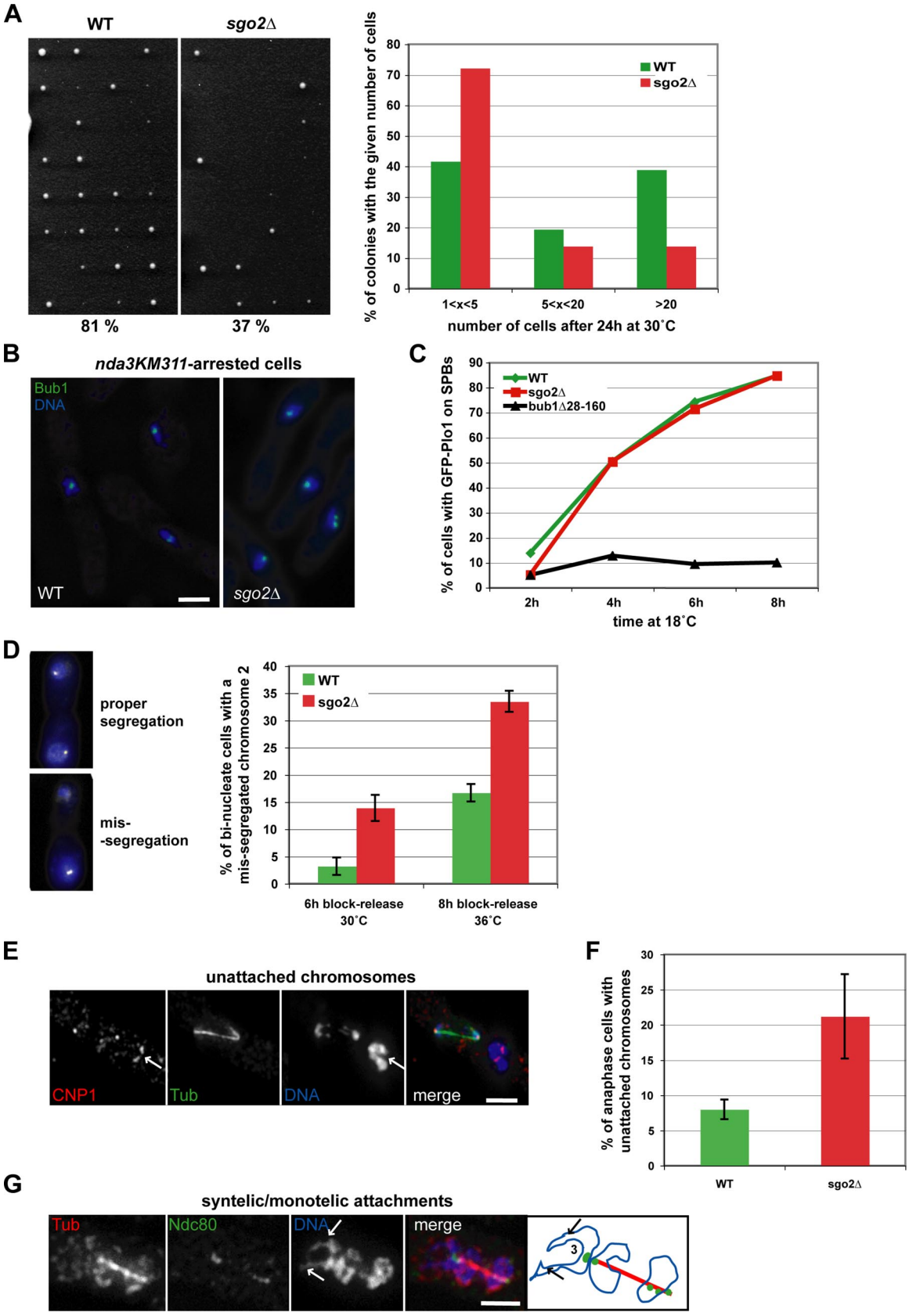

Figure 1. Sgo2 is required for proper chromosome biorientation after a prolonged spindle checkpoint arrest. (A) Cells lacking Sgo2 die after a *nda3KM311*-dependent arrest. Cells were shifted to 18°C for 8 h, and then individual cells were isolated on plates (rich medium) and left at 30°C for 3 d. The numbers under the pictures indicate the corresponding cell viability for that particular experiment (% of isolated cells forming colonies). The right panel quantifies the number of cells per individual colony after 24 h at 30°C. (B) *nda3KM311* and *sgo2*Δ

fects. In meiosis I, Sgo2 is required to ensure mono-orientation of sister-chromatids (Rabitsch *et al.*, 2004; Vaur *et al.*, 2005). In mitosis, cells lacking Sgo2 show no visible defect in chromosome segregation (Rabitsch *et al.*, 2004), but they nonetheless lose chromosomes at a significant rate and exhibit sensitivity to the microtubule-depolymerizing drug TBZ (Kitajima *et al.*, 2006). Taken together, these data suggest an as yet unidentified role of Sgo2 in the faithful segregation of chromosomes in mitosis.

Here we demonstrate that fission yeast Shugoshin2 (Sgo2) is crucial for chromosome biorientation in the first anaphase after a prolonged spindle checkpoint arrest, likely through regulating the Passenger proteins. Indeed, Sgo2 colocalizes with the Passenger proteins in early mitosis and promotes their efficient recruitment onto centromeres and telomeres. Sgo2 also regulates the stability of the Survivin-INCENP interaction. Sgo2 localization on centromeres is itself dependent on Survivin. Taken together our results suggest that Sgo2 either regulates, or is itself, a docking site for the recruitment of the Passenger proteins on centromeres.

MATERIALS AND METHODS

Yeast Strains and Mitotic Arrests

A list of the strains used in this study is provided in Supplementary Information (Supplementary Table S1). Cells were arrested in metaphase using overexpression of Mad2 as previously described (Vanoosthuyse *et al.*, 2004). *nda3-KM311* cells (~5–7.10^6 cells per ml of rich media) were shifted to 18°C for typically 6–8 h. The synchrony upon release was greater when cells were arrested for 6 h and released at 30°C. At 8 h, hyper-condensed chromosomes were more often individualized and had the tendency to move away from each other (especially chromosome 3, the smallest of all three chromosomes in *Schizosaccharomyces pombe*). In these conditions there was greater chromosome loss, even in the wild-type (see Figure 1).

Microscopy

Yeast cells with green fluorescent protein (GFP)-, cyan fluorescent protein (CFP)-, or mCherry-tagged proteins (Snaith, 2005) were grown in rich media and very briefly fixed in 100% methanol (<30 s) before observation, unless stated otherwise. Preliminary experiments have demonstrated that the methanol fixation did not affect the localization pattern of the proteins of interest (not shown). Imaging was performed using an Intelligent Imaging Innovations (3i) Marianas system (Denver, CO). This system uses a Zeiss Axiovert fluorescence microscope (Thornwood, NY), a CoolSNAP HQ charge-coupled device camera (Photometrics, Woburn, MA), and Slidebook software (3i; Photometrics). Unless otherwise stated, images were deconvolved using the no-neighbor algorithm. For immunofluorescence, cells were fixed for 5–15 min by the addition of freshly prepared paraformaldehyde solution (3%).

Coimmunoprecipitations

Coimmunoprecipitations were carried out as previously described (Vanoosthuyse *et al.*, 2004). When stated, immunoprecipitated proteins were dephosphorylated using λ-phosphatase (New England Biolabs, Beverly, MA) according to the manufacturer's instructions. Immunoprecipitated proteins were analyzed by Western blot and when stated were quantified using the ImageQuant software (GE Healthcare, Little Chalfont, United Kingdom). In Figure 5D1, quantifications were performed on five blots corresponding to five independent experiments.

RESULTS

Sgo2 Is Required for Faithful Chromosome Segregation after a Prolonged Spindle Checkpoint Arrest

We first investigated the functional consequences of deleting *Sgo2* (*sgo2*Δ) on chromosome segregation. As reported earlier, lack of Sgo2 has only a mild effect on chromosome segregation in cycling, unchallenged cells (roughly 1% of cells mis-segregate chromosome 2; Kitajima *et al.*, 2004 and not shown). However, we found that *sgo2*Δ cells struggled to recover from an early mitotic arrest induced by the cold-sensitive β-tubulin mutant *nda3-KM311*. Unable to form a spindle in the cold, *nda3KM311* cells generate unattached kinetochores in mitosis that are recognized by the spindle checkpoint (Hiraoka *et al.*, 1984). The viability of *sgo2*Δ *nda3-KM311* cells decreased by 40–50% after 8 h at 18°C (not shown and Figure 1A). However this was not due to a spindle checkpoint defect because *sgo2*Δ *nda3-KM311* cells arrested normally in early mitosis before cytokinesis (Figure 1, B and C, and not shown). Indeed chromosomes hyper-condensed in *sgo2*Δ cells as in *sgo2*+ cells and <2% of cells had septa (not shown). Furthermore, kinetochores recruited normal levels of the spindle checkpoint kinase Bub1 (Figure 1B), Polo and Cdc2 kinases accumulated on spindle pole body (SPBs; Figure 1C, data not shown), and cohesion at centromeres was maintained (Supplementary Figure 1). We conclude from these observations that Sgo2 is not required for a spindle checkpoint arrest triggered by the lack of attachment of kinetochores to microtubules.

To understand why *nda3-KM311* cells lacking Sgo2 (*nda3KM311 sgo2*Δ) die after the arrest, we monitored the first mitosis after the release. The *nda3-KM311*–dependent arrest is reversible by shifting the temperature back to 30–36°C. This allows the spindle to be reformed and the cells enter fairly synchronously into anaphase (Kanbe *et al.*, 1990 and *Materials and Methods*). The timing of anaphase onset was similar in wild-type and in *sgo2*Δ cells (Supplementary Figure 2). However we observed a significant increase in

Figure 1 (facing page). *nda3KM311* cells arrested at 18°C for 8 h recruit a similar amount of the spindle checkpoint kinase Bub1 on kinetochores. Scale bar, 2 μm. (C) *nda3KM311* GFPplo1, *sgo2*Δ *nda3KM311* GFP-plo1, and *bub1*Δ*20-160 nda3-KM311* GFPplo1 cells were incubated at 18°C for 8 h, and the number of metaphase cells was scored, using the strong enrichment of GFPplo1 on SPB after SPB duplication (Mulvihill *et al.*, 1999). Cut12-CFP was used as a SPB marker for confirmation (not shown). Wild-type and *sgo2*Δ cells accumulated metaphase cells with similar kinetics after the shift at 18°C. On the contrary, the checkpoint-deficient allele *bub1*Δ*28-160* (Vanoosthuyse *et al.*, 2004) did not show any significant accumulation of metaphase cells. Furthermore in all backgrounds except *bub1*Δ*28-160* chromosomes remained hyper-condensed and Cdc13/cyclinB and Cdc2 levels were maintained on SPBs (not shown). (D) *nda3KM311* and *sgo2*Δ *nda3KM311* cells were shifted at 18°C for either 6 or 8 h to arrest them in early mitosis. They were then released at 30 or 36°C, respectively, to allow anaphase onset, and the segregation of chromosome II was monitored in binucleate cells using GFP-tagged chromosomes (Ding *et al.*, 2004). A minimum of 800 binucleate cells has been counted in total after a minimum of three independent experiments. (E) Example of anaphase with unattached chromosomes observed in the absence of Sgo2 upon release from an *nda3KM311*-dependent arrest. Cells were shifted at 18°C for 8 h, released at 36°C for 10 min, and then fixed with paraformaldehyde and processed for immunofluorescence with antibodies recognizing tubulin (tub) or the kinetochore component CNP1. The arrow indicates the two pairs of unattached sister-centromeres. Scale bar, 2 μm. (F) Quantification of the previous. (G) Example of monotelic/syntelic attachment. See diagram on the right of the picture for explanation. In that particular example, chromosome 3 (labeled "3" on the diagram) is easily recognizable by the two rDNA protrusions it carries (arrows). The two unseparated kinetochores of chromosome 3 are found at the left pole. Consistent with this, the four other kinetochores (chromosomes 1 and 2) are found on the spindle, two at the poles and two lagging. Finally, the fact that the 2 rDNA protrusions of chromosome 3 are facing away from the spindle is the final argument to conclude that chromosome 3 has not segregated. Compare to E for a normal anaphase segregation of chromosome 3 with its rDNA protrusions segregating in between the two poles. Scale bar, 2 μm.

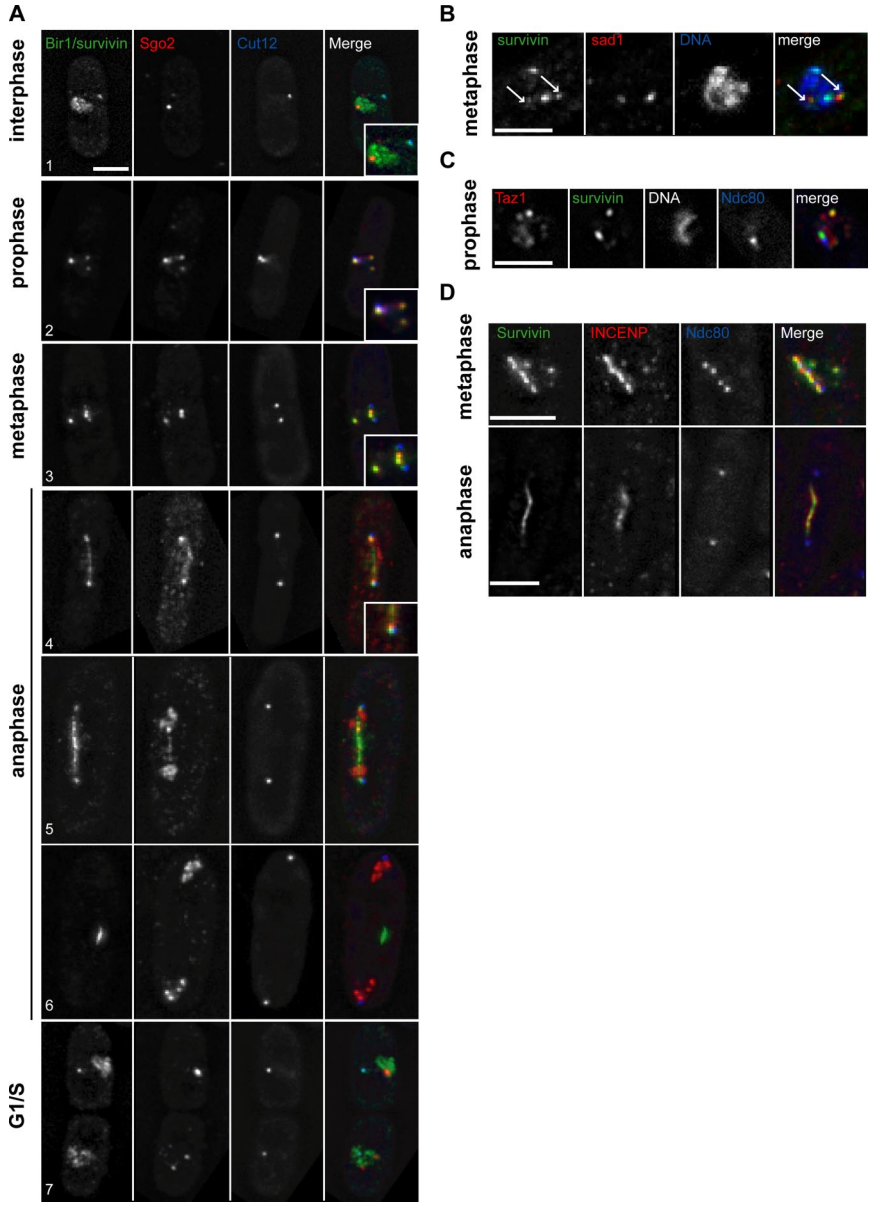

Figure 2. Sgo2 and the Passenger proteins colocalize in early mitosis before anaphase. (A) Sgo2-mCherry, GFP-Bir1/Survivin, and the SPB component Cut12 were observed concomitantly. To do so, we used a strain expressing 1) Sgo2 as a fusion with the monomeric red fluorescent protein mCherry (Shaner *et al.*, 2004) (Sgo2-mCH), 2) the Spindle Pole Body (SPB) component Cut12 as a fusion with CFP (Cut12-CFP) and 3) Bir1/Survivin fused to GFP (GFP-Bir1/Survivin). All protein fusions were integrated at the endogenous locus and are functional. Cells 1–7 were selected from a cycling population and are representative of each individual stage of the cell cycle. Blow-ups of panels 1–4 are shown in the merge channel. Note that in early anaphase (panel 4) Survivin and Sgo2 showed greatly reduced colocalization at the poles. Survivin localization was consistent with a localization at the SPBs, whereas Sgo2 most likely localized on centromeres. (B) Bir1/Survivin localizes on SPBs every mitosis, as shown by its colocalization with the SPB component Sad1. (C) Bir1/Survivin localizes on telomeres every mitosis, as shown by its colocalization with the telomere component Taz1. (D) GFP-Bir1/Survivin and Pic1/INCENP-mCherry colocalize at all stages of the cell cycle. Examples of metaphase and anaphase are shown. Ark1/Aurora B displayed the same localization pattern (not shown and Morishita *et al.*, 2001). Scale bar, 3 μm in all panels.

chromosome mis-segregation in the absence of Sgo2 (up to 35% of cells mis-segregate chromosome II when cells were maintained in the arrest for 8 h and subsequently released at 36°C; see Figure 1D and *Materials and Methods*). Consistent with this, a significant proportion of cells proceeded through anaphase with clear kinetochore-microtubule attachment defects (Figure 1, E–G): 1) some cells proceeded into anaphase even though one or two chromosome(s) were still unattached and 2) some kinetochores appeared to show monotelic/syntelic attachments (because it is difficult to observe single microtubules in *S. pombe,* it is hard to show conclusively whether both kinetochores of a pair are attached or not). These segregation defects are consistent with inefficient chromosome biorientation.

Sgo2 Colocalizes with Chromosomal Passenger Proteins in Early Mitosis

One major regulator of both kinetochore-microtubule function and the spindle checkpoint are the Chromosomal Passenger Proteins (see Vagnarelli and Earnshaw, 2004 for review). Interestingly, *sgo2*Δ cells display a synthetic lethal interaction with *cut17*.275, a mutant allele of the Passenger protein Survivin, supporting the idea that Sgo2 and the Passenger proteins act together to ensure accurate chromosome segregation (not shown). To test the possibility that Sgo2 might regulate the Chromosomal Passenger proteins, we first examined their localization pattern concomitantly throughout the cell cycle. In interphase, Sgo2 usually localized at one to three distinct foci that colocalize with the DNA but not with Cut12 (Figure 2A, panel 1). These foci actually represent the heterochromatic regions present at telomeres (a detailed analysis of Sgo2 function in interphase is to be published elsewhere). A fainter signal also colocalized with the whole DAPI mass, suggesting that Sgo2 might also associate with chromatin. In interphase, Passenger proteins were enriched in the nucleolus and on the SPB-centromere cluster (Supplementary Figure 3A). In early mitosis, however, Sgo2 and Bir1/Survivin colocalized to two to three

Table 1. Summary of Bir1/Survivin and Sgo2 localization throughout the cell cycle

Stage of the cell cycle	Bir1/Survivin	Sgo2
Interphase	Nucleolus + SPB-centromere cluster	Telomeres
Prophase	SPB-centromere cluster + telomeres	SPB-centromere cluster + telomeres
Metaphase	SPB + centromeres + telomeres	Centromeres + telomeres
Early anaphase	SPB + spindle	Centromeres
Late anaphase	Spindle midzone	Chromatin
G1/S transition	Nucleolus + SPB-centromere cluster	Telomeres
nda3KM311-arrested cells	Centromeres	Centromeres

The sites affected in the absence of Sgo2 (*sgo2*Δ) are underlined.

distinct foci associated with the DNA, on either side of the nucleus (Figure 2A, panel 2): one focus was in close association with the SPB-centromere cluster, whereas the other foci represented the clustered telomeres, as shown by their colocalization with the telomere component Taz1 (Figure 2C). In metaphase, Sgo2 and Bir1/Survivin still colocalized, at least partially (Figure 2A, panel 3): they were both found on centromeres and telomeres, but Bir1/Survivin also localized on SPBs, as shown by its colocalization with Cut12 and Sad1 (Figure 2B). This localization pattern has been confirmed by arresting the cells in metaphase by overexpressing the spindle checkpoint component Mad2 (He *et al.,* 1997; Supplementary Figure 3B). In early anaphase (Figure 2A, panels 4 and 5), Bir1/Survivin and Sgo2 displayed very little colocalization: the bulk of Bir1/Survivin was found on the two SPBs and the spindle, whereas Sgo2 remained on centromeres (see blowup in Figure 2A, panel 4). Occasionally Sgo2 was also found on the spindle in the early stages of anaphase, as reported earlier (Rabitsch *et al.,* 2004). Later in anaphase, Sgo2 left centromeres to associate with the whole chromosomes, whereas Bir1/Survivin concentrated on the spindle midzone (Figure 2A, panel 6). In septating cells (G1/S transition, Figure 2A, panel 7), Sgo2 and Bir1/Survivin both adopted their interphase localization pattern. In conclusion, Sgo2 and Bir1/Survivin have distinct and very complex localization patterns throughout the cell cycle, and they only colocalize in the early stages of mitosis before anaphase onset. A summary of their localization pattern is presented in Table 1. Note that in cycling cells, all three Passenger proteins Bir1/Survivin, Pic1/INCENP, and Ark1/Aurora B colocalized (see Morishita *et al.,* 2001; Huang *et al.,* 2005; Figure 2C and not shown). This detailed in vivo analysis of Passenger proteins demonstrates that the localization pattern published previously is incomplete: our data show that in interphase they localize not only in the nucleolus but also on the SPB-centromere cluster and that they localize on centromeres, telomeres, and SPBs every mitosis.

Sgo2 Regulates the Chromosomal Passenger Complex Localization on Centromeres and Telomeres in Early Mitosis

Our localization studies have shown that Bir1/Survivin and Sgo2 colocalize on chromosomes during the early stages of mitosis, where kinetochore-microtubule attachments are established. Furthermore, Figure 1 shows that Sgo2 has a role in chromosome biorientation after a prolonged spindle checkpoint arrest. We therefore investigated the possibility that Sgo2 and the Passenger proteins could regulate one another.

Previous chromatin immunoprecipitation (ChIP) studies have shown that both Sgo2 and Bir1/Survivin bind to the inner-centromere (outer heterochromatic repeats [OTR]; see Morishita *et al.,* 2001; Kitajima *et al.,* 2004). Therefore, we tested whether the lack of Sgo2 would affect the localization of the Passenger proteins on centromeres. Note that the localization pattern of Sgo2 and the Passenger proteins are significantly affected by prolonged activation of the spindle checkpoint: in early mitosis of a cycling population, the Passenger proteins normally localize to SPBs, centromeres, and telomeres (Table 1), whereas Sgo2 localizes to centromeres and telomeres. In *nda3KM311*-arrested *sgo2+* cells, Sgo2 and all three Passenger proteins become enriched only on centromeres (Figure 3, A and B). Lack of Sgo2 had a dramatic effect on the localization of all three Passenger proteins: in *nda3KM311*-arrested cells lacking Sgo2, no more than two Bir1/Survivin foci were observed, and Bir1/Survivin was also found in the nucleus (Figure 3, C and D). The two foci corresponded to SPBs, as shown by their colocalization with the SPB marker Cut12 (Figure 3D). In 80% of the cells Pic1/INCENP and Ark1/Aurora B localized on SPBs and in the nucleus. In the remaining 20% of the cells, Pic1/INCENP and Ark1/Aurora B could still be observed on centromeres, but at a dramatically reduced level (estimated at a 10-fold reduction by quantification of the fluorescence; Figure 3E and not shown). In conclusion, Sgo2 is crucial for the localization and/or maintenance of all three Passenger proteins on centromeres upon checkpoint arrest. This is consistent with the results described in Figure 1, showing that Sgo2 is required to ensure proper chromosome biorientation upon recovery from a prolonged checkpoint arrest.

We next analyzed the localization pattern of the Passenger proteins in the absence of Sgo2 in cycling cells. First, the lack of Sgo2 had no effect on the localization of the Passenger proteins on the spindle (not shown). Second, the lack of Sgo2 abolished their telomere localization in early mitosis (Figure 3F and not shown). Third, the absence of Sgo2 affected the centromeric localization of the three Passenger proteins, but in different ways. Lack of Sgo2 had a striking effect on the centromeric localization of Bir1/Survivin in the early stages of mitosis: in more than 65% of metaphase *sgo2*Δ cells (39/60 early mitotic cells analyzed after 3D capture and deconvolution), Bir1/Survivin failed to localize on centromeres and concentrated on SPBs instead (Figure 3G). In 10% of the cells, Bir1/Survivin prematurely transferred to the spindle. In the remaining 25% of the cells, Bir1/Survivin remained on centromeres, albeit at a reduced level. Interestingly, lack of Sgo2 had a less severe effect on the localization of the two other Chromosomal Passenger proteins Pic1/INCENP and Ark1/Aurora B in cycling cells: their localization at centromeres was maintained in more than 65% of the cells (Figure 3G). To determine whether Ark1/Aurora B was recruited to centromeres with the same efficiency in the absence of Sgo2, two

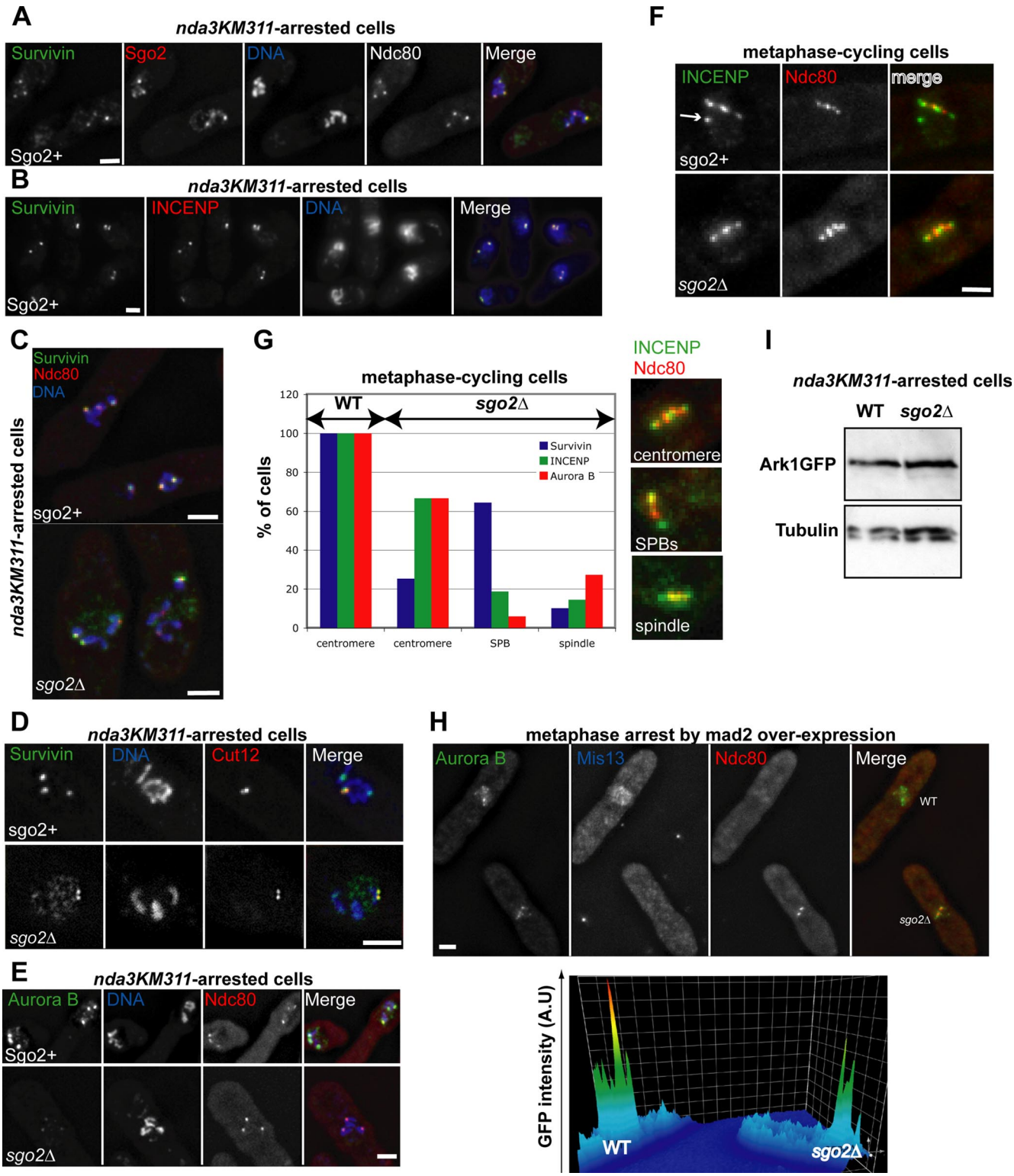

Figure 3. Lack of Sgo2 affects the localization of the Passenger proteins. Scale bar, 2 μm in all panels. (A) Bir1/Survivin and Sgo2 localize only on centromeres in *nda3KM311*-arrested cells. (B) Bir1/Survivin and Pic1/INCENP colocalize on centromeres in *nda3KM311*-arrested cells. (C) In the absence of Sgo2, Bir1/Survivin failed to localize on centromeres in *nda3KM311*-arrested cells and instead colocalized with the SPB marker Cut12 (D). Note that in both C and D Bir1/Survivin was also found in the nucleus in the absence but not in the presence of Sgo2. (E) *nda3KM311* cells expressing Ark1/Aurora B-GFP and the kinetochore marker Ndc80-CFP were arrested at 18°C in the presence or the absence of the *Sgo2+* gene. Only cells where the three kinetochore pairs were clearly individualized were examined (3D analysis and deconvolution on 55 cells). In these cells, it was straightforward to determine whether Ark1/Aurora B localized on centromeres or on SPBs. In the absence of *Sgo2+* (*sgo2*Δ), Ark1/Aurora B was clearly mislocalized on SPBs in 80% of the cells but remained on centromeres at a drastically reduced level in 20% of the cells. The pictures *Sgo2+* and *sgo2*Δ were taken in exactly the same conditions. (F) In the absence of

populations of cells expressing Ark1/Aurora B-GFP (with and without a functional *Sgo2+* gene) were arrested in metaphase by overexpression of the spindle checkpoint component Mad2. Both populations were mixed and imaged at the same time. To differentiate between the two types of cells, the kinetochore component Ndc80 was tagged with CFP in *sgo2Δ* cells, whereas the kinetochore component Mis13 was tagged with mCherry in *Sgo2+* cells. By analyzing the fluorescence intensities of the GFP signals in both populations, imaged in the same fields of view, we were able to show that lack of Sgo2 induces a reduction of 40–50% in the amount of Ark1/Aurora B recruited on centromeres in cells arrested in metaphase by the overexpression of Mad2 (Figure 3H). However the total amount of Ark1/Aurora B in the cell was not affected by lack of Sgo2, as shown by Western blot analysis (Figure 3I and see also Figure 5). In conclusion, Sgo2 is also an important regulator of the Chromosomal Passenger proteins in cycling cells. Interestingly, lack of Sgo2 had a more penetrant effect on Bir1/Survivin localization.

To test how specific an effect the lack of Sgo2 was having on overall centromere structure, we analyzed the localization of a number of other proteins important for kinetochore-microtubule attachments. Lack of Sgo2 did not affect the localization of the centromere-specific histone H3-variant CENP-A/Cnp1 (Figure 1E), the spindle checkpoint component Bub1 (Figure 1B), the kinetochore component Ndc80 (Figures 1G and 3, C and F) and the Ask1 member of the DASH complex (Supplementary Figure 4A). Furthermore we detected no alleviation of silencing of a reporter gene inserted in the centromeric heterochromatin (outer heterochromatic repeats *OTR::ade6+*) in the absence of Sgo2, suggesting that lack of Sgo2 does not affect heterochromatin structure and function (Supplementary Figure 4B). Taken together, these data show that lack of Sgo2 does not affect centromere and kinetochore functions per se and demonstrate that the *sgo2Δ* effect on the localization of the Passenger proteins is specific.

Sgo2 Localization Pattern Is Affected in the Bir1/Survivin Mutant bir1.46

Recently it has been shown that centromeric localization of the *Drosophila* Shugoshin homologue MEI-S332 is dependent on the Passenger protein INCENP (Resnick *et al.,* 2006). To determine whether Sgo2 localization was regulated by the Passenger proteins, we analyzed the localization pattern of Sgo2 in a Bir1/Survivin temperature-sensitive mutant (*bir1.46*, where Bir1/survivin is mutated on residue 976 [C976Y], Huang *et al.,* 2005). As reported previously (Huang *et al.,* 2005), we found that INCENP did not localize on centromeres anymore at the restrictive temperature of 36°C (not shown). In these cells, at either the permissive temperature of 25°C or the restrictive temperature of 36°C, we could not detect Sgo2 localization on centromeres (Figure 4). We conclude that Sgo2 localization on centromeres is dependent on Survivin.

However, the other allele of Bir1/Survivin (*cut17.275*, where Bir1/survivin is mutated on residue 990 (A990T; Morishita *et al.,* 2001) had a far less penetrant effect on the localization of Sgo2, Aurora B, and INCENP (Supplementary Figure 5). Moreover, *cut17.275* experienced a transient Mad2-dependent delay at the restrictive temperature of 36°C (Supplementary Figure 5). We suggest that *cut17.275* is a weaker allele of Bir1/Survivin than *bir1.46*.

Sgo2 Regulates the Stability of the Pic1/INCENP-Bir1/Survivin Complex

In vertebrates the Passenger proteins form a stable complex, the CPC (reviewed in Vader *et al.,* 2006b). However in fission yeast, the fact that lack of Sgo2 has a more penetrant effect on Bir1/Survivin localization than on the other Passenger proteins brings into question the idea that the three Chromosomal Passenger proteins form a single stable "trimeric" complex, while on centromeres. To address this issue further, we undertook a biochemical analysis of the complexes formed by the Chromosomal Passenger proteins in the presence or absence of Sgo2. To do this, we fused a C-terminal S-ZZ tag (Cheeseman *et al.,* 2001) to all four proteins Ark1/Aurora B, Bir1/Survivin, Pic1/INCENP and Sgo2. These proteins all replace the endogenous proteins and are expressed at their native loci from their own promoters. They are stable and detectable on a Western blot, although they appear to be present at different levels, consistent with them having independent roles in the cell (Figure 5A). We cannot however rule out that these differences are not in part due to different transfer efficiencies. We noticed that Pic1/INCENP-SZZ was cleaved artifactually during immunoprecipitation (Figure 5, C and D, and not shown). Therefore coimmunoprecipitation experiments were not carried out for more than 40 min to avoid the complete cleavage of Pic1/INCENP in the extract. In these experiments, we failed to detect an interaction between Bir1/Survivin and Ark1/Aurora B (see below). However, the formation of a complex between Ark1/Aurora B and Pic1/INCENP on one hand and Pic1/INCENP and Bir1/Survivin on the other hand was clearly detectable (Figure 5B). Interestingly, lack of Sgo2 did not affect complex formation between Ark1/Aurora B and Pic1/INCENP (Figure 5C), but the complex between Pic1/INCENP and Bir1/Survivin was consistently found at reduced levels in mitotic checkpoint-arrested cells (in 10 experiments, see Figure 5D). We observed a two- to fivefold reduction in the amount of Bir1/Survivin recovered after Pic1/INCENP immunoprecipitation in the absence of Sgo2 compared with wild type. Interestingly, the effect of Sgo2 on the Survivin-INCENP complex was specific to checkpoint-arrested cells where all three proteins localize on centromeres (Figure 3). Lack of Sgo2 had no significant effect on the Survivin-INCENP complex in interphase cells (Figure 5D2), where Sgo2 does not colocalize with Survivin or INCENP (Figure 2).

Note that we failed to detect the trimeric complex Survivin-INCENP-Aurora B found in vertebrate cells, where

Figure 3 (facing page). Sgo2, none of the Passenger proteins localize on telomeres in early mitosis. A representative metaphase cell expressing Pic1/INCENP-GFP and the kinetochore marker Ndc80-CFP is shown in the presence (*sgo2+*) or in the absence of Sgo2 (*sgo2Δ*). Note that no signal outside the spindle axis (arrow) is observed in the absence of Sgo2. (G) Centromere localization of the Passenger proteins in the absence of Sgo2 in a normal mitosis. Metaphase *sgo2Δ* cells of a cycling population were analyzed by 3D capture and deconvolution. Three types of cells were identified (representative pictures of cells expressing Pic1/INCENP-GFP and Ndc80-CFP are placed on the right of the histogram). In the first type, the Passenger protein localized on centromeres; in the second type, it localized on SPBs, and in the third type, it localized prematurely on the spindle. The histograms show the percentage of cells found in each category for each of the three Passenger proteins. (H) In metaphase of cells lacking Sgo2, centromeres recruit only half the wild-type amount of Ark1/Aurora BGFP (see text for details). (I) Ark1/Aurora B-GFP is stable in the absence of Sgo2. Whole cell extracts of *Sgo2+* or *sgo2Δ* cells arrested using the *nda3KM311* mutation were analyzed by Western blot. Tubulin provided the loading control.

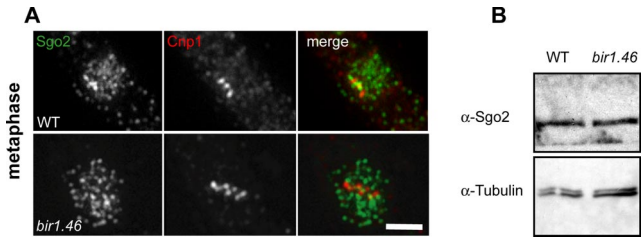

Figure 4. Sgo2 localization on centromeres is abolished by the Bir1/Survivin mutation *bir1.46*. (A) *bir1.46* cells expressing Sgo2-GFP were grown overnight at 25°C, shifted or not at the restrictive temperature of 36°C, and then processed for immunolocalization of Sgo2 (α-GFP antibody) and the kinetochore marker Cnp1 (α-CNP1 antibody, kind gift of Prof. R. Allshire). In metaphase cells of *bir1.46* cells, Sgo2 did not concentrate on centromeres and instead localized in the whole nucleus at both temperatures. Scale bar, 2 μm (B) The *bir1.46* mutation did not affect the levels of Sgo2 in the cell, as shown by Western blot with our anti-Sgo2 antibody. Tubulin was used as a loading control.

INCENP act as a bridge between Survivin and Aurora B (for recent data, see Gassmann *et al.*, 2004; Klein *et al.*, 2006; Vader *et al.*, 2006a). Our data suggest that this trimeric complex, if it exists in fission yeast, is not very abundant or stable, as we can readily detect a complex between Survivin and the full-length INCENP on one hand and an INCENP-Aurora B complex on the other hand (Figure 5). The in vitro cleavage of INCENP might also prevent us from detecting this trimeric complex, as INCENP would fail to act as a bridge between Survivin and Aurora B. Together, these observations suggest that the Passenger proteins form several independent complexes in fission yeast.

To test whether Sgo2 directly interacts with Passenger proteins, we used conventional coimmunoprecipitation and tandem affinity purifications followed by mass spectrometry analysis. However, despite intense efforts, we have so far failed to find reproducible evidence of a stable Sgo2-Passenger protein complex (not shown and see *Discussion*).

The Conserved C-Terminus Domain of Sgo2 Is Crucial for Its Localization

Sgo2 can be divided into three domains: a conserved N-terminal coiled-coil region, an internal domain rich in hydroxylated residues, and finally a conserved C-terminus (Rabitsch *et al.*, 2004). The conserved N-terminus of human Sgo1 has been shown to bind microtubules (Salic *et al.*, 2004). The function of the conserved C-terminal domain of Sgo2 is unknown. We therefore deleted it (*sgo2Δ563-647*). The truncated protein was stable (Figure 6A). Unlike full length Sgo2, it was not enriched on telomeres in interphase, but instead decorated all chromatin (Figure 6B). In early mitosis, *sgo2Δ563-647* still localized on centromeres in 90% of cells, but localized on telomeres in only 10% of cells (Figure 6C and not shown). Strikingly, *sgo2Δ563-647* was no longer found on centromeres upon *nda3KM311*-dependent arrest (Figure 6D, the arrest itself was unaffected). Instead *sgo2Δ563-647* relocalized to the whole nucleus. Furthermore, Bir1/Survivin was targeted to SPBs, suggesting that Sgo2 needs to be on centromeres for Bir1/Survivin to be targeted to centromeres (Figure 6D). Thus, upon checkpoint arrest, *sgo2Δ563-647* cells had a very similar phenotype to *sgo2Δ* cells. Consistent with this, *sgo2Δ563-647* and *sgo2Δ* cells exhibited a similar amount of chromosome loss upon release (13.9 ± 1.95% for *sgo2Δ* and 18 ± 3.95% for *sgo2Δ563-647*, a minimum of 700 cells counted). Thus the conserved C-terminus is required for Sgo2 localization on centromeres in checkpoint-activated cells, but is apparently dispensable in a normal unchallenged mitosis. These observations suggest that the conserved C-terminus is required to maintain Sgo2 (and therefore the Passengers) on centromeres when mitosis is unusually delayed/arrested. Another possible interpretation is that the affinity of the centromere for Sgo2 is different in cycling cells versus checkpoint-arrested cells.

DISCUSSION

This is the first detailed analysis of Shugoshin2 (Sgo2) function in fission yeast mitosis. We demonstrate that Sgo2 is required for efficient targeting of the Chromosomal Passenger proteins to centromeres in early mitosis. Sgo2 becomes essential for centromeric localization of the Passengers during a spindle checkpoint arrest triggered by the tubulin mutant *nda3KM311*. On release from this arrest, cells lacking Sgo2 exhibit significant chromosome biorientation defects. These defects lead to chromosome mis-segregation and a marked decrease in cell viability.

How Does Sgo2 Control Passenger Protein Localization?

It has been demonstrated in vertebrates that the complex formed between Survivin, Borealin, and INCENP is crucial for the targeting of the Passenger proteins to centromeres (Klein *et al.*, 2006). Borealin has the ability to bind to chromatin in vitro (Klein *et al.*, 2006), and it stabilizes the complex between Survivin and INCENP in vivo (Gassmann *et al.*, 2004; Klein *et al.*, 2006; Vader *et al.*, 2006a). Creating a protein fusion between Survivin and INCENP bypasses the requirement for Borealin, confirming that stability of the Survivin-INCENP complex is a key determinant of Passenger localization on centromeres (Vader *et al.*, 2006a). We have shown that lack of Sgo2 destabilizes the Pic1/INCENP-Bir1/Survivin complex and impairs the recruitment of the Passenger proteins to centromeres. Therefore, fission yeast Sgo2 appears to play a similar role to Borealin in vertebrate cells.

In HeLa cells, Survivin forms oligomers on centromeres (Klein *et al.*, 2006). If that were true in fission yeast, our observations could be interpreted in at least three ways: either 1) Sgo2 is a cofactor that increases the stability of the Bir1/Survivin-Pic1/INCENP complex, or 2) lack of Sgo2 prevents a core Bir1/Survivin-Pic1/INCENP-Ark1/Aurora B complex from recruiting additional Bir1/Survivin molecules, or finally 3) Sgo2 recruits Bir1/Survivin to a preformed and perhaps independently targeted INCENP-Aurora B complex to generate a trimeric complex (Survivin-INCENP-Aurora B) that is more stable on centromeres than the INCENP-Aurora B complex on its own.

As yet we have failed to demonstrate a stable protein interaction between Sgo2 and the Passenger proteins. This could be for technical reasons, as both Bir1/Survivin and Pic1/INCENP are susceptible to proteolysis. In addition, the Sgo2 interaction with the Passengers might be very dynamic and/or take place only on chromosomes, either of which would make it very difficult to analyze biochemically. FRAP (fluorescence recovery after photobleaching) experiments have been attempted to compare the dynamics of fission yeast Passengers proteins with one another and with Sgo2. Because of low signals Sgo2 has so far proven difficult to analyze. Preliminary results show that Bir1/Survivin and Pic1/INCENP both turnover rapidly at fission yeast centromeres but that they display different dynamics (not shown). Vertebrate Survivin is known to be a far more dynamic

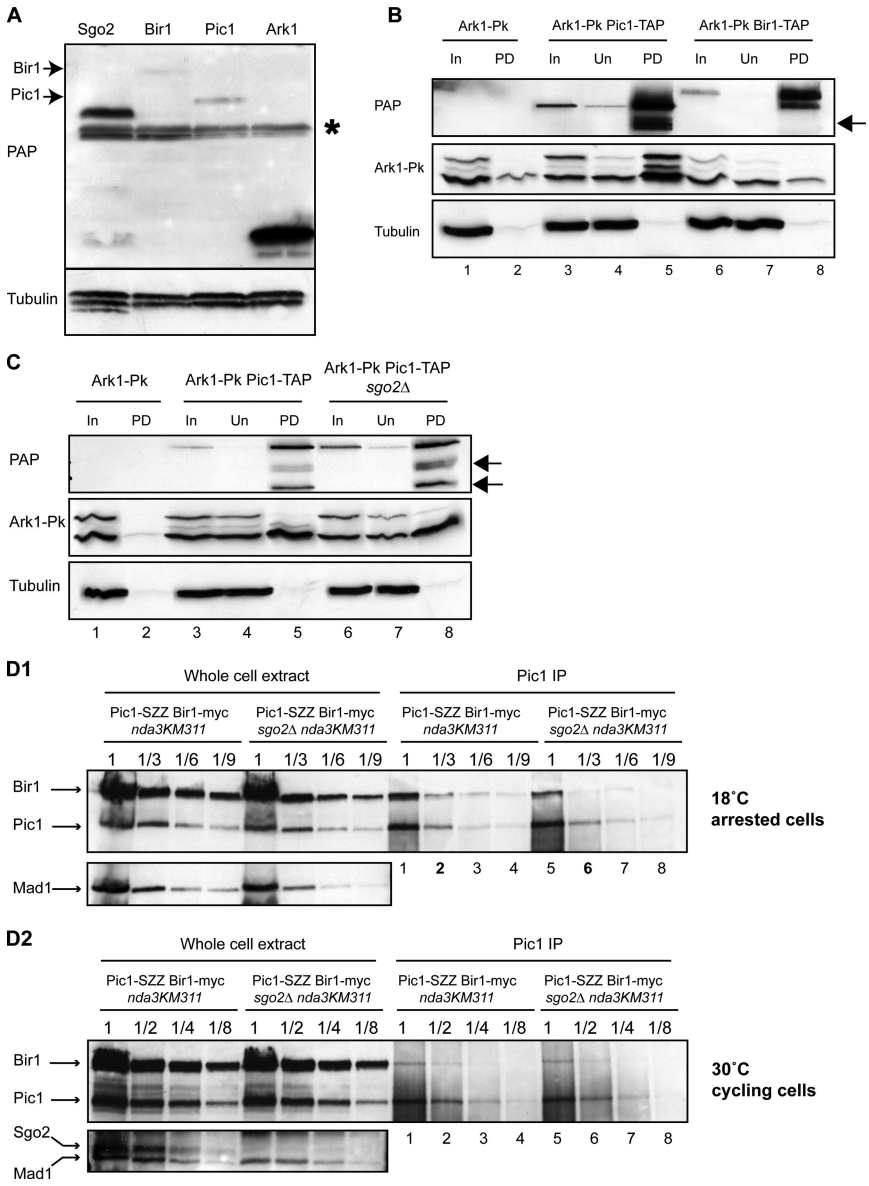

Figure 5. Lack of Sgo2 specifically destabilized the complex between Bir1/Survivin and Pic1/INCENP in checkpoint-arrested cells. (A) Ark1/Aurora B, Pic1/INCENP, Bir1/Survivin, and Sgo2 were all tagged with a SZZ tag. Whole cell extracts of each tagged strains were processed by Western blot, and the levels of expression of each construct were assessed with the Peroxidase anti-Peroxidase (PAP) antibody that recognizes the ZZ tag. Tubulin provided a loading control. The asterisk highlights a protein recognized aspecifically by the PAP antibody. (B) Ark1/Aurora B coimmunoprecipitates with Pic1/INCENP, but not with Bir1/Survivin. Bir1/Survivin-SZZ or Pic1/INCENP-SZZ was pulled down on IgG Sepharose beads from *nda3-KM3111*-arrested cells. The complexes immunoprecipitated were assessed by Western blot for the presence of Ark1/Aurora B tagged with the Pk epitope (Ark1/Aurora B-Pk). A little Ark1/Aurora B-Pk sticks to the IgG beads even in the absence of any SZZ-tagged protein in the extract (see lane 2). The amount of Ark1/Aurora B-Pk pulled down by the IgG beads is dramatically increased in the presence of Pic1/INCENP-SZZ but not in the presence of Bir1/Survivin-SZZ (compare lanes 5 and 8). (C) Lack of Sgo2 does not affect the stability of the Pic1/INCENP-Ark1/Aurora B complex. Pic1/INCENP-SZZ was pulled down on IgG Sepharose beads from *nda3-KM3111*-arrested cells. The complexes immunoprecipitated were assessed by Western blot for the presence of Ark1/Aurora B tagged with the Pk epitope (Ark1/Aurora B-Pk). There is consistently no significant difference in the amount of Ark1/Aurora B-Pk pulled down by Pic1/INCENP in the presence or absence of Sgo2 (compare lanes 5 and 8). The experiments described in B and C have been repeated at least four times. For B–D: In, input; Un, unbound; PD, pull-down. The arrows indicate Pic1/INCENP degradation products that occurred during the 40-min immunoprecipitation. These degradation products are not found in the total extracts (IN) or in extracts prepared from methanol-fixed cells (not shown). (D) Lack of Sgo2 reduces the interaction between Pic1/INCENP and Bir1/Survivin in checkpoint-arrested cells (D1) but not in interphase cells (D2). Pic1/INCENP-SZZ was pulled down as in B with the exception that IgG dynabeads were used instead of IgG Sepharose beads. The complexes immunoprecipitated were assessed for the presence of Bir1/Survivin tagged with the myc epitope (Bir1/Survivin-myc) using a polyclonal rabbit anti-myc antibody (A14, Santa Cruz Biotechnology, Santa Cruz, CA). The same antibody also recognized the TAPTAG on Pic1/INCENP. (D1) Extracts were prepared from cells arrested in mitosis at 18°C using the tubulin mutation *nda3KM311*. Serial dilutions of both extracts and immunoprecipitated complexes were loaded on the gel. Compare particularly lanes 2 and 6. We consistently observed a 2–5-fold reduction in the amount of Bir1/Survivin recovered in the Pic1/INCENP immunoprecipitated complexes in the absence of Sgo2. This experiment has been carried out 10 times and a significant reduction was observed every time. In this particular example, we observed a threefold reduction in the level of Bir1/Survivin recovered when lanes 2 and 6 were quantified. Despite a slight underloading of the *sgo2*Δ extracts, we chose to show this particular example because the amount of Pic1/INCENP recovered in the immunoprecipitated complexes was very similar between the Sgo2+ and *sgo2*Δ extracts. Lack of Sgo2 did not consistently affect the stability of either Pic1/INCENP or Bir1/Survivin (see D2 and Supplementary Figure 6). (D2) The same experiment as in D1 was performed with protein extracts made from cycling cells (30°C). Here, lack of Sgo2 had no effect on the amount of Bir1/Survivin recovered after Pic1/INCENP immunoprecipitation. In D1 and D2, the checkpoint component Mad1 was used as a loading control. In D2, extracts were also probed with an antibody directed against Sgo2 to confirm that the extracts were Sgo2+ or *sgo2*Δ.

member of the Passenger proteins on centromeres than Aurora B (Delacour-Larose *et al.*, 2004), suggesting that local regulation of individual Passenger proteins occurs on centromeres. Two distinct models take account of such properties: 1) Survivin could interact with Sgo2 and form a dynamic subcomplex of the Passenger proteins with important targeting functions, or 2) Sgo2 could influence Passenger protein localization indirectly, most simply through regulation of their centromere docking site. In this model, destabilization of the Survivin-INCENP complex in the absence of Sgo2 is a secondary consequence of docking site mis-regulation (Figure 7A).

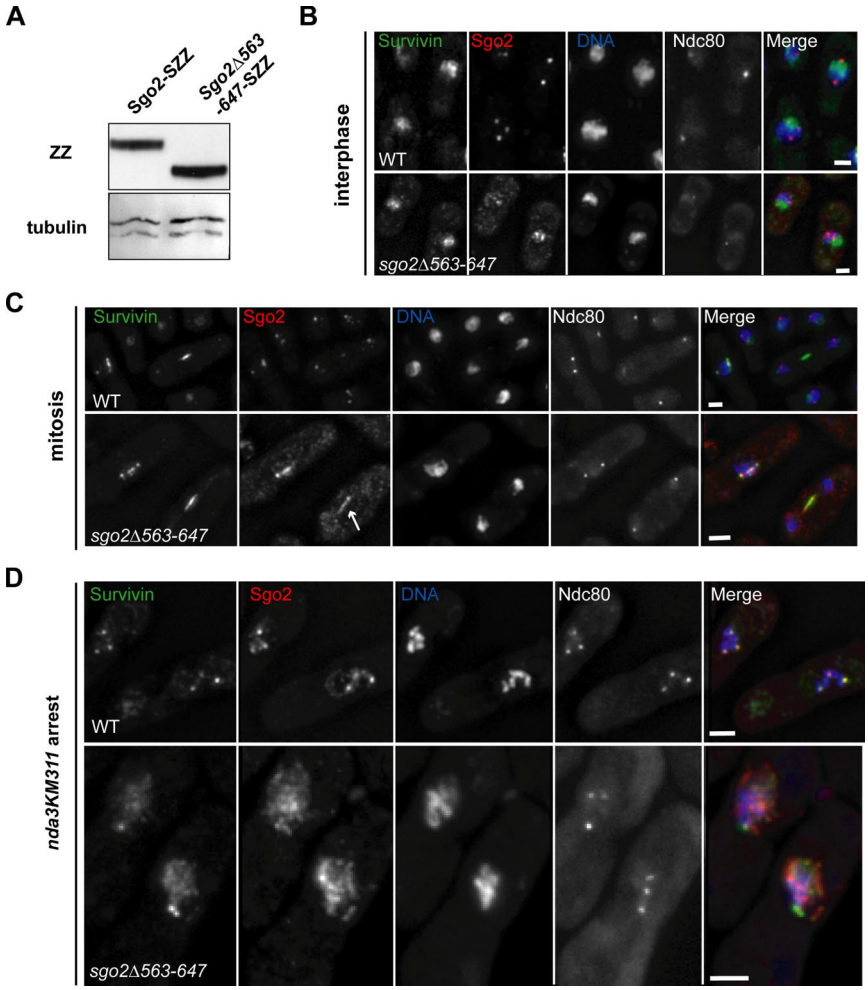

Figure 6. The conserved C-terminus of Sgo2 is required for Sgo2 localization on centromeres upon checkpoint arrest. Scale bar, 2 μm in all panels. (A) The truncated protein Sgo2Δ563-647 is stable. Whole cell extracts of the indicated strains were analyzed by Western blot. Tubulin provided a loading control. (B) The truncated protein Sgo2Δ563-647 was tagged with mCherry, and its localization pattern was analyzed in cycling cells concomitantly with GFP-Bir1/Survivin and the kinetochore marker Ndc80CFP. Strikingly the truncated protein was not enriched on telomeres in interphase but instead was found over the chromatin. (C) In mitosis of a cycling population, neither GFP-Bir1/Survivin nor Sgo2Δ563-647 localized on telomeres. However, they still localized on centromeres. Interestingly in anaphase, the truncated protein Sgo2Δ563-647 localized on the spindle (arrow). This is consistent with the idea that the conserved N-terminus of the Shugoshin family is a microtubule-binding domain (Salic *et al.*, 2004). (D) In *nda3KM311*-arrested cells Sgo2Δ563-647 localized exclusively in the nucleus and no more than two GFP-Bir1/Survivin foci were detected, indicating that GFP-Bir1/Survivin localized on SPBs (see Figure 2).

Why Is Sgo2 More Important after a Spindle Checkpoint Arrest?

Although lack of Sgo2 affects Passenger protein localization on centromeres every mitosis (Figure 3), this leads to only a slight increase in chromosome loss (roughly 1% of binucleate cells mis-segregate chromosome 2; not shown and Kitajima *et al.*, 2004). However, upon checkpoint activation lack of Sgo2 has dramatic consequences (Figures 1 and 2). What is special about chromosome segregation after this spindle checkpoint arrest? In *nda3KM311*-arrested cells, we can distinguish three types of kinetochore pairs: 1) some have no connection with either SPB, 2) some show monotelic or syntelic attachments, and finally 3) some are already amphitelically attached (Supplementary Figure 7A). Furthermore, the duplicated SPBs often move away from each other (Grishchuk and McIntosh, 2006) and, as reported previously, the nuclear envelope can lose its circular shape and ultimately form separate vesicles (Kanbe *et al.*, 1990; Grishchuk and McIntosh, 2006; Supplementary Figure 7B). These observations suggest that the geometry created during the *nda3-KM311* arrest (kinetochore-SPBs distance + nuclear shape) constrains kinetochore capture by microtubules when the spindle reforms (see Figure 7B). We propose that during recovery from this arrest efficient Passenger proteins function is required to correct kinetochore-microtubule attachments, until proper biorientation of all chromosomes is achieved. Passenger proteins function may also be required to prolong the arrest until biorientation is achieved, but our data (Supplementary Figure 2) suggest that such a delay is brief as no significant difference in the timing of anaphase onset was observed between wild-type and *sgo2*Δ cells.

In a normal mitosis there are fewer geometric constraints on kinetochore-microtubule attachments and weakened Passenger protein function would be tolerated by most cells. Therefore the role of Sgo2 in ensuring the efficient recruitment of the Passenger proteins on centromeres is especially important after a prolonged *nda3KM311*-dependent arrest.

Why Is the Effect of Sgo2 on Passenger Protein Localization More Pronounced in a Spindle Checkpoint Arrest?

Although lack of Sgo2 has a mild effect on the localization of the Passenger proteins in a normal mitosis, Sgo2 becomes indispensable upon checkpoint activation. We can interpret this observation in three ways that are not mutually exclusive: 1) Sgo2 could regulate the maintenance of the Passenger proteins on centromeres. Cells lacking Sgo2 might be able to maintain sufficient Passenger proteins on centromeres in a normal mitosis (which lasts only a few minutes in fission yeast), whereas Sgo2 might be required to maintain the Passengers on centromeres during a prolonged mitosis (several hours in the case of an *nda3KM311*-dependent arrest). 2) In addition to Sgo2 there could be another factor (referred to as X, see Figure 7A) that recruits the Passenger

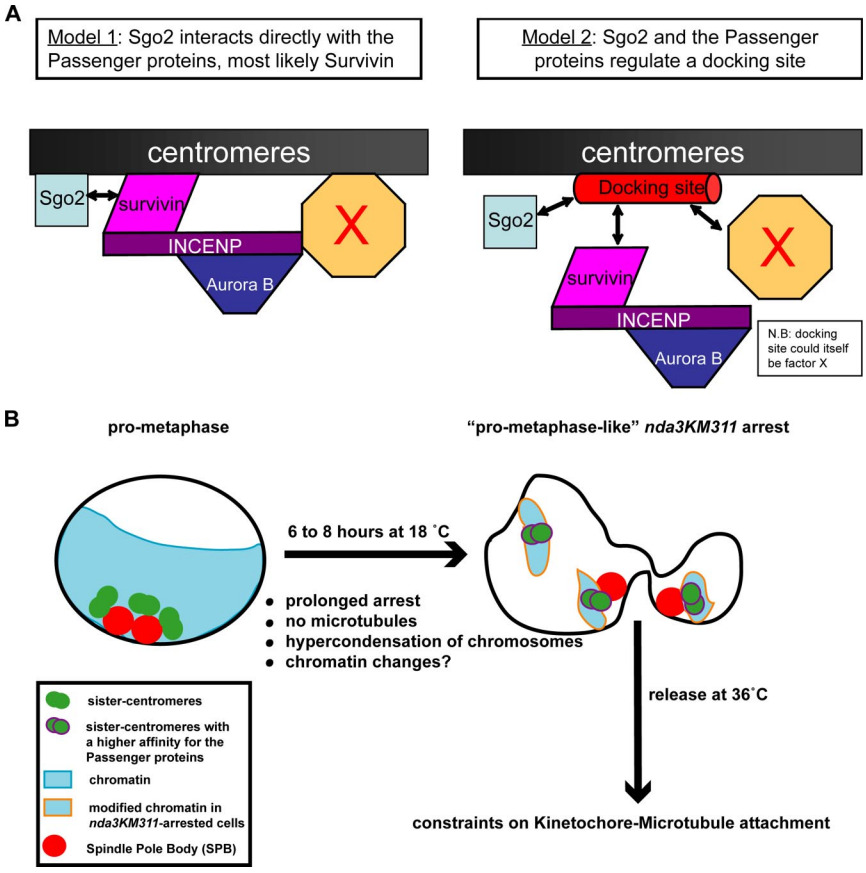

Figure 7. (A) Models for Sgo2 function. In the first model, Sgo2 forms a complex with Survivin. This complex, together with an additional factor X (see text for details) are crucial for Passenger proteins targeting to centromeres. The arrows indicate that Survivin and Sgo2 are interdependent for their localization on centromeres. In the second model, Sgo2 does not interact directly with the Passenger proteins but instead regulates their docking site. The stable interaction between the Passenger proteins and their docking site is in turn crucial for Sgo2 localization. (B) Diagram highlighting the wide range of changes in *nda3KM311*-arrested cells compared with normal prometaphase cells. At the restrictive temperature of 18°C, chromosomes hypercondense, the nuclear envelope loses its circular shape, and duplicated SPBs move away from each other, maintaining or not connections with one or more kinetochores. Furthermore, we speculate that chromatin changes (highlighted by a yellow line around the chromosomes) could enhance the association of the Passenger proteins with centromeres (see *Discussion*). On release at 36°C, the nuclear geometry generated in the arrest constrains kinetochore-microtubules attachments.

proteins to centromeres. Although factor X can recruit the Passenger proteins to centromeres in a normal mitosis in the absence of Sgo2, it is unable to compensate for loss of Sgo2 in an *nda3KM311* arrest. To explain this, we speculate that factor X acts in a microtubule-dependent manner. In budding yeast, the Cdc14 phosphatase regulates the centromere to spindle transfer of passenger proteins in anaphase (Pereira and Schiebel, 2003), and in fission yeast, the Cdc14-related phosphatase Clp1/Flp1 has been shown to regulate Ark1/Aurora B localization on centromeres (Trautmann *et al.*, 2004). However Clp1 localizes on centromeres in *nda3KM311*-arrested cells (Trautmann *et al.*, 2004), so we think it is unlikely that Clp1 is factor X. 3) Finally the interaction of the Passenger proteins with centromeres is qualitatively different in *nda3KM311*-arrested cells (possibly due to chromatin modifications; see below) and Sgo2 is necessary for this interaction.

Passenger Protein Localization Is Affected in nda3KM311-arrested Cells

Interestingly, the localization pattern of the Passenger proteins is affected by activation of the spindle checkpoint. In early mitosis of a cycling population, the Passenger proteins localize to SPBs, centromeres and telomeres (Table 1); however in an *nda3KM311*-dependent checkpoint arrest (a prometaphase-like mitotic arrest), they concentrate exclusively on centromeres (Figure 3, A and B). This indicates that the interaction of the Passenger proteins with centromeres is enhanced relative to telomeres and SPBs in *nda3KM311*-arrested cells. On recovery from checkpoint arrest, the Passenger proteins relocalize to telomeres and SPBs (data not shown), suggesting that the process of "exclusion" from telomeres and SPBs is reversible. This behavior is reminiscent of the bromodomain protein BrD4 in HeLa cells. It has recently been reported that BrD4 is released from chromatin upon activation of the spindle checkpoint but relocalizes to chromatin upon recovery from the arrest (Nishiyama *et al.*, 2006). The release of BrD4 from chromatin coincides with changes in the acetylation status of specific histone residues. These observations suggest that chromatin is modified during prolonged mitotic arrest and that these changes modify the chromatin association of a subset of proteins. It is therefore possible to envisage that the association of the Passenger proteins with telomeres is destabilized upon checkpoint activation by such chromatin modifications or that their centromere association is enhanced.

Conservation of Sgo2 Function(s)?

It would appear that Sgo2 has conflicting roles in meiosis and in mitosis in fission yeast. In meiosis I, Sgo2 is required to maintain sister-chromatid mono-orientation (Vaur *et al.*, 2005), whereas our data demonstrate that Sgo2 helps ensure proper biorientation of sister-chromatids in mitosis. One way to reconcile these apparent differences would be to propose that in meiosis I Sgo2 actually helps to maintain biorientation of the homologues and hence mono-orientation of the sister-chromatids. Whether this is compatible with a role of Sgo2 in recruiting Passenger proteins onto meiotic centromeres remains to be elucidated.

Arabidopsis thaliana, Zebrafish, *Mus muculus*, and *Homo sapiens* also have a Shugoshin2 homologue (Rabitsch *et al.*, 2004; Watanabe, 2005). So far there are conflicting reports regarding the role of human hSgo2: one suggests an involvement for hSgo2 in chromosome biorientation, which is con-

sistent with our results (Watanabe and Kitajima, 2005). However, a later report argues that hSgo2 is involved in the protection of centromeric cohesion in mitosis by recruiting the phosphatase PP2A to centromeres (Kitajima *et al.*, 2006).

The mitotic functions of fission yeast Sgo2 appear similar to the mitotic functions of budding yeast Sgo1. Indeed deletion of ScSgo1 also triggers biorientation defects upon release from a spindle checkpoint arrest (Indjeian *et al.*, 2005). It has also been suggested that ScSgo1 is crucial to activate the spindle checkpoint in response to lack of tension at kinetochores (Indjeian *et al.*, 2005). Interestingly, it has recently been shown in budding yeast that the Survivin-INCENP complex was required to link centromeres to microtubules, independently of Aurora B. However, that linkage could provide local regulation of Aurora B in response to lack of tension at kinetochores (Sandall *et al.*, 2006). To explain how ScSgo1 is required to sense lack of tension at kinetochores, it would be interesting to know whether ScSgo1, like SpSgo2 in fission yeast, is able to regulate the Survivin-INCENP complex.

It has recently been demonstrated that the centromeric localization of MEI-S332, the *Drosophila* Shugoshin homologue, is under the control of the Chromosomal Passenger proteins INCENP and Aurora B and that this is important to protect centromeric cohesion in meiosis I (Resnick *et al.*, 2006). Together with our results, this demonstrates that multiple members of the Shugoshin family interact with Chromosomal Passengers. We suggest that Sgo-Passenger protein interactions will regulate distinct processes, including cohesion, biorientation and microtubule dynamics, in a wide range of model systems and in different developmental contexts.

CONCLUSION

Here we have shown in fission yeast that Shugoshin2 (Sgo2) is an important regulator of Passenger proteins localization on centromeres and telomeres specifically in early mitosis. Functional interactions between Sgo2 and the Passenger proteins become essential in conditions that challenge kinetochore-microtubule interactions, such as those after prolonged microtubule depolymerization.

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